DeCREASED VACUOLAR ACIDIFICATION CAPACITY IN DRUG-RESISTANT RAT LIVER PRENEOPLASTIC NODULES

Göran N. Andersson, Ulla-Britta Torndal, and Lennart C. Eriksson
Department of Pathology, Karolinska Institutet, Huddinge University Hospital, S-141 86 Huddinge, Sweden

ABSTRACT

Rat liver nodules produced by intermittent 2-acetylaminofluorene feeding exhibit alterations in cell surface receptors reminiscent of impairment of vacuolar acidification. In this report, vacuolar acidification activity, measured as the ATP-dependent quenching of acridine orange, was characterized in liver and nodular membrane fractions using various ion-transport inhibitors and with respect to nucleotide specificity and divalent cation dependence. Based on these criteria and on the comparison of vacuolar acidification activity with mitochondrial, lysosomal, and plasma membrane marker enzymes in different subcellular fractions, it was concluded that the assay measures the proton pump associated with exocytic and/or endocytic vacuolar compartments. When the vacuolar acidification activity was compared in liver and nodular subcellular membrane fractions, it was found that the vacuolar acidification was most strongly reduced in nodular low-density membrane fractions enriched in Golgi-derived membranes and endocytic vesicles. The data indicate that vacuolar, i.e., exocytic and/or endocytic, prelysosomal intracellular compartments in rat liver nodules are markedly deficient in acidification capacity, possibly providing an explanation to various metabolic aberrations, such as diminished iron accumulation and reduced protein degradation, observed in rat liver nodular cells.

INTRODUCTION

In processes of chemical hepatocarcinogenesis, populations of cells develop that are resistant to the cytotoxic effects of carcinogens and other xenobiotics (1, 2). In rat liver, these resistant cells proliferate in the presence of promoters, such as 2-AAF, which are mitoinhibitory to normal hepatocytes and form nodules by clonal expansion of the resistant population (3, 4). One interesting feature of cells forming nodules is the altered regulation of certain cell surface receptor-mediated functions such as transferrin-mediated iron uptake (5) and uptake of desialylated glycoproteins (6–8). These nodular cells resemble, in this respect, cultured mutant cells which have been selected for survival by drugs which elevate the pH within intracellular endocytic vesicles and lysosomes (9–12). Compared to wild-type cells, such acidification-deficient mutants not only survive comparatively higher doses of exposition to the selecting agent but also commonly to a variety of structurally unrelated toxins.

There is now a large body of evidence that the interior of both the endocytic and exocytic vacuolar apparatus maintains an acidic pH due to the activity of vacuolar-type proton-translocating ATPases (13–16). In the exocytic process, an acidic environment has been shown to be required for certain post-translational processing reactions associated with secretion, such as proteolytic cleavage of proproteins and glycosylation, and also to be required for proper sorting of membrane and secretory constituents (17). In the endocytic process, the progressively increasing acidity of endocytic compartments has been shown to be important for proper ligand dissociation and receptor recycling processes (17).

In view of the striking similarities between acidification-deficient mutant cells and rat liver nodular cells, the present study deals with the characterization and quantification of the vacuolar acidification activity in subcellular organelles isolated from rat liver nodules. Our data show that proton pumping in intracellular membrane compartments involved in exocytosis and/or endocytosis is markedly reduced in nodules and suggest that cells forming nodules are severely deficient in vacuolar acidification. The nodular system thus appears to be an attractive in vivo model to study the role of acidic vacuolar membrane compartments in the carcinogenic process.

MATERIALS AND METHODS

Materials. Acridine orange, DCCD, monensin, oligomycin, ouabain, NEM, ATP, GTP, CTP, and UTP were purchased from Sigma. Histidine, sodium orthovanadate, and sodium azide were from Merck. Percoll was purchased from Pharmacia and Metrizamide from Nyegaard. DCCD, oligomycin, and monensin were prepared as stock solutions in absolute ethanol and serially diluted with ethanol to appropriate concentrations to avoid the final ethanol concentration in the incubation mixture exceeding 0.5%. NEM was prepared fresh daily. The monomeric form of orthovanadate was prepared by boiling a 10 mM solution in NaOH at pH 10 until the yellow color vanished.

Methods. Male Wistar rats (Möllegaards Breeding Center, Ejby, Denmark) were used for all experiments. Liver nodules were produced by intermittent feeding of a diet containing 0.05% 2-acetylaminofluorene (18). Rats selected for the experiments had an abundance of medium-sized nodules, around 5 mm in diameter, and were used after termination of the 25-wk experimental protocol. The rats had then been off the 2-AAF diet for at least 2 wk. As controls, age-matched rats which had been fed standard laboratory rat chow were used.

For subcellular fractionation, the rats were starved overnight and anesthetized by an i.p. injection of sodium hexabarbital. Details concerning liver perfusion and harvesting of nodules are described in Ref. 18. In one set of experiments, the homogenate was fractionated into a crude nuclear (300 × g, 10 min) pellet, ML (5,000 × g, 15 min) pellet, and microsomal (105,000 × g, 60 min) pellet fractions according to the method of Wattiaux et al. (19) using a discontinuous Metrizamide density gradient. Plasma membranes were isolated on Percoll according to Loten and Redshaw-Loten (20). Mitochondria were prepared by the method of Sottocasa et al. (21). Marker enzymes for the various organelles were determined as previously described (5, 18).

Proton pump activity was measured with acridine orange as described by Stone et al. (22). Due to rapid inactivation, the activity was measured on the day of preparation of the subcellular fractions.

The standard reaction mixture contained: 30 mM histidine, pH 7.0; 20 mM KCl; 130 mM NaCl; 50- to 500-μM fractions of protein; 2.5 μM...
acridine orange; 10 \mu M oligomycin; 1 mM ouabain; 100 \mu M orthovanadate; and 3 mM MgCl₂ in a final volume of 1.5 ml. The reaction was initiated by adding ATP to a final concentration of 1.3 mM. The concentrations of various substrates, cofactors, and inhibitors are indicated in the respective legends. NEM was preincubated with the membranes for 10 min at room temperature prior to the addition of divalent cation and ATP. The activity was recorded as the difference in absorbance at 492 to 540 nm using an Aminco DW 2a dual wavelength spectrophotometer. The values of proton pump activity are expressed as the decrease in absorbance (initial slope value)/min/mg of protein. Data are presented as the mean ± SD of 3 to 5 separate experiments.

RESULTS

In order to characterize the proton-translocating ATPase in a crude microsomal fraction from normal liver and liver nodules, various known ion-transport inhibitors were used (Table 1). It was found that neither oligomycin (10 \mu M), sodium azide (1 mM), ouabain (1 mM), nor sodium orthovanadate (100 \mu M) to any significant extent inhibited ATP-dependent acridine orange quenching in microsomes from liver or nodules. This rules out that proton pumping in this fraction was contributed from mitochondrial F₁F₀-ATPase, which is completely inhibited at 0.1 \mu M oligomycin (23), or from Na⁺,K⁺-ATPase [complete inhibition at 100 \mu M ouabain (23)], or other plasma membrane ATPases which form phosphorylated intermediates and which are inhibited at 1 \mu M orthovanadate (23). On the other hand, NEM, DCCD, and the ionophore monensin inhibited proton translocation with half-maximal inhibition attained at 5 to 7 \mu M, 1.1 to 1.3 \mu mol/mg of protein, and 0.9 to 1.3 nmol/mg of protein, respectively. No significant differences in the sensitivity to these inhibitors were apparent when liver and nodules were compared (Table 1). In the case of monensin and DCCD, it was found necessary to relate the effective inhibitory dose to the amount of membrane protein used for the incubation rather than to the final concentration in the reaction tube.

The nucleotide specificity and cation preference to promote proton translocation were investigated (Table 2). It was found that, of the four nucleotide triphosphates tested (ATP, GTP, CTP, and UTP), only ATP effectively supported acidification. ADP had no effect. The apparent \( K_a \) for ATP was determined from double-reciprocal plots and was found to be 1.3 mM. However, ATP concentrations in excess of 1.5 mM inhibited proton pump activity in a dose-dependent manner (data not shown). Of the different divalent cations tested, Mg²⁺ was slight more effective than Mn²⁺, whereas Fe²⁺ and Zn²⁺ were much less effective (Table 2). Ca²⁺ had no effect.

Omission of KCl and NaCl in the reaction mixture reduced the activity by 60%, and addition of either salt alone at 75 mM restored the activity (data not shown). This indicates that the presence of chloride, possibly as a counterion, is required for maximum efficiency of this proton pump.

As the initial characterization and quantification of the proton-translocating ATPase were carried out using a crude microsomal fraction, containing endoplasmic reticulum, Golgi membranes, plasma membrane, endocytic vesicles, and possibly coated vesicles, it was of interest to investigate the subcellular site(s) of the activity in both liver and nodules. In the first experiment (Fig. 1), total cellular membranes (TPF) were separated on the basis of size differences into nuclear (N), mitochondrial-lysosomal (ML), and microsomal (P) fractions by differential centrifugation (6). Compared to liver, nodules exhibited a 50% reduction of acidification activity in the microsomal (P) fraction, whereas a 35% increase was seen in the mitochondrial-lysosomal (ML) fraction. In the nuclear (N) fractions, no difference between the two tissues was observed. In the nodular total particulate fraction a more modest, or 15%, reduction compared to liver was noted. However, taking into consideration the differences in protein content of liver com-

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**Table 1** Effect of inhibitors on ATP-dependent acidification

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Liver</th>
<th>Nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligomycin</td>
<td>10 \mu M</td>
<td>95</td>
<td>110</td>
</tr>
<tr>
<td>Oubain</td>
<td>1000 \mu M</td>
<td>85</td>
<td>114</td>
</tr>
<tr>
<td>Ortovanadate</td>
<td>1000 \mu M</td>
<td>103</td>
<td>114</td>
</tr>
<tr>
<td>Na₅</td>
<td>1000 \mu M</td>
<td>93</td>
<td>85</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>0.5 \mu M</td>
<td>95</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>5.0 \mu M</td>
<td>65</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>50 \mu M</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dicyclohexylcarbodiimide</td>
<td>0.015 \mu mol/mg</td>
<td>107</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>0.15 \mu mol/mg</td>
<td>84</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>1.5 \mu mol/mg</td>
<td>37</td>
<td>48</td>
</tr>
<tr>
<td>Monensin</td>
<td>1.25 nmol/mg</td>
<td>91</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>1.0 nmol/mg</td>
<td>62</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>2.0 nmol/mg</td>
<td>23</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2** Effect of nucleotides and divalent cations on acidification

<table>
<thead>
<tr>
<th>Additions</th>
<th>Liver</th>
<th>Nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (+Mg²⁺)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GTP (+Mg²⁺)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CTP (+Mg²⁺)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UTP (+Mg²⁺)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mn²⁺ (+ATP)</td>
<td>74</td>
<td>95</td>
</tr>
<tr>
<td>Fe²⁺ (+ATP)</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>Zn²⁺ (+ATP)</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>Ca²⁺ (+ATP)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Distribution of ATP-dependent acidification activity in subcellular fractions from rat liver and nodules. The homogenate was fractionated according to the size of the membrane particles as described (6), and ATP-dependent acridine orange quenching was determined as described in the legend to Table 2. Data are from three fractionation experiments. Columns, mean; bars, SD; N, nuclear; P, microsomal; C, control liver; N, nodules.
pared to nodular TPF (Table 3), the activity on a liver wet weight basis was reduced to 68% in nodules compared to liver.

To investigate whether the observed changes in the subcellular distribution of the vacuolar acidification activity in nodules compared to liver could be explained by differences in organelle sedimentation rates, the TPF and microsomal fractions were analyzed for the activity of marker enzymes representing mitochondria (succinate cytochrome c reductase), endoplasmic reticulum (NADPH cytochrome c reductase), lysosomes (acid phosphatase), and the Golgi complex (galactosyltransferase). As seen in Table 3, the ratios of nodule to liver activities for all the enzymes analyzed were in the range of 0.92 to 1.1. The acidification activity, on the other hand, was reduced to 0.85 (TPF) and 0.5 (microsomes) in nodules compared to liver (Fig. 1). It is concluded from this experiment that no general differences in organelle sedimentation rates exist between liver and nodules.

In order to further localize the site(s) of the reduced expression of the acidification activity in nodular microsomes, this fraction was subjected to sucrose density gradient centrifugation. This gradient step enables the separation by flotation of a LDMF fraction containing mainly endoplasmic reliculum with plasma membranes and endocytic vesicles (5, 24, 25), an IMF, containing cis-Golgi (25) and endocytic vesicles (24), and finally a RM fraction containing mainly endoplasmic reticulum with trace contamination of plasma membranes (5, 25). The distribution patterns of mitochondrial, lysosomal, and plasma membrane marker enzymes in these microsomal subfractions are shown in Table 4. When these fractions were analyzed for acidification activity (Fig. 2), the highest enrichment in normal liver was seen in the IMF fraction (4-fold), followed by the LDMF fraction (2.5-fold). No enrichment was seen in the RM fraction compared to the total membrane fraction (TPF). In nodules the situation was significantly different, since no enrichment was observed in the IMF and LDMF fractions compared to the TPF fraction. Thus, the pump activity in these two fractions was reduced to around one-fourth of the values detected in the corresponding liver fractions. The activity in the nodular RM fraction was reduced to 60% of the control.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Succinate-cytochrome c reductase</th>
<th>Acid phosphatase</th>
<th>Adenylate cyclase</th>
</tr>
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<tbody>
<tr>
<td>TPF</td>
<td>69 ± 12* (35)*</td>
<td>124 ± 44 (2.3)</td>
<td>8 ± 3 (2.6)</td>
</tr>
<tr>
<td>RM</td>
<td>5.4 ± 0.9 (2.8)</td>
<td>32 ± 11 (0.6)</td>
<td>8 ± 1 (2.6)</td>
</tr>
<tr>
<td>IMF</td>
<td>3.6 ± 0.9 (1.9)</td>
<td>199 ± 30 (3.7)</td>
<td>33 ± 4 (11)</td>
</tr>
<tr>
<td>LDMF</td>
<td>0.4 ± 0.2 (0.2)</td>
<td>434 ± 35 (8.0)</td>
<td>14 ± 4 (4.6)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>193 ± 6 (100)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>ND</td>
<td>5430 ± 239 (100)</td>
<td>ND</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>ND</td>
<td>ND</td>
<td>304 ± 8 (100)</td>
</tr>
</tbody>
</table>

* Cytchrome c reduced (nmol/min/mg protein).  
* Nitrophenol formed (nmol/min/mg protein).  
* Cyclic AMP formed (nmol/min/mg protein).  
* Mean ± SD of 3 to 5 subcellular fractions.  
* Numbers in parentheses, relative specific activity compared to the most purified fraction.  
* ND, not determined.

DISCUSSION

The expression of cell surface receptors is assumed to determine the ability of the cell to respond to environmental signals regulating cellular behavior. Major alterations in receptor activity have been noted during different functional states of the cell, such as in regeneration, fetal and neonatal growth, as well as during tumor development.

The roles of intracellular acidic compartments with respect to regulation of cell surface receptor activity have been elucidated in various ways, for example, by exposing cells to weak...
bases such as ammonium chloride, chloroquine, or primaquine; by dissipating proton gradients using ionophores such as monensin (26); and furthermore by characterization of acidification-deficient mutants (9–12).

With respect to the transferrin and ASGP receptor systems, it has been noticed that HepG2 hepatoma cells exhibit diminished iron uptake (27) and decreased ASGP receptor levels at the cell surface (28) when challenged with weak bases. These receptor alterations have been suggested to be the result of impaired ligand dissociation due to the elevated vacuolar pH caused by these drugs (29, 30). In the acidification mutants, decreased iron uptake via transferrin receptors has been a regular finding (11, 12, 31).

We have recently studied transferrin and asialoglycoprotein receptors in rat liver nodules (5, 6). These cells express much elevated levels of transferrin receptor but, despite this upregulation, they show a marked decrease in the rate of iron accumulation (5). This is consistent with and may provide a molecular explanation to earlier findings that the nodular cells are iron deficient and, in addition, resistant to iron loading (32, 33). In the case of ASGP receptors, nodular cells express a considerable reduction of cell surface and intracellular receptors and also exhibit a slower-than-normal rate of intracellular metabolism of the ligand (6). Furthermore, nodular cells express only the ASGP receptor monomer, in contrast to normal liver cells where higher molecular weight oligomeric species are also found (6).

In view of the similarities of nodular cells and cells with impaired vacuolar acidification, it was of interest to clarify whether nodular cells are deficient in vacuolar acidification expressed at the level of the proton-translocating ATPase. The acidification activity, measured as the quenching of acridine orange, was initially characterized using various known ion transport inhibitors and with respect to substrate specificity and divalent cation dependence. No inhibition of acridine orange quenching was seen in the presence of high concentrations of ouabain, orthovanadate, or oligomycin, indicating that the activity measured in a microsomal fraction is not due to mitochondrial or plasma membrane-associated ion pump activities (22, 23). The acidification activity was, on the other hand, sensitive to N-ethylmaleimide, dicyclohexylcarbodiimide, and sodium azide with half-maximal inhibition values comparable to vacuolar ATPases in platelet-dense granules (23), rat liver multivesicular bodies (34), endocytic vesicles (10, 35), and coated vesicles from rat liver and brain (22, 36, 37). It can thus be concluded that this assay measures the vacuolar-type, proton-translocating ATPase. Substrate specificity studies demonstrated that ATP could support the proton pumping, but not GTP, UTP, or CTP. It is known that the lysosomal proton pump is active in the presence of GTP as well as ATP (38, 39), whereas the pump associated with Golgi and endocytic compartments can only use ATP (10, 40). Based on this evidence and on the distribution profile of lysosomes and mitochondria compared to acidification activity (cf. below) in the microsomal subfractions, it is furthermore concluded that the assay measures the proton pump associated with exocytic or endocytic, i.e., vacuolar compartments.

When the acidification activity in liver and nodular subcellular fractions was compared, it was found that the vacuolar acidification capacity in a total membrane fraction was only slightly reduced in nodules compared to liver. A more significant reduction in nodules was observed when microsomal fractions were compared, whereas the acidification activity in ML fractions was increased in nodules. This indicates a relocalization phenomenon presumably due to altered targeting and/or turnover of the proton pump.

The subcellular distribution of vacuolar acidification activity was further studied in microsomal subfractions. In liver, the highest enrichment was seen in an IMF followed by a LDMF. Both these fractions contain Golgi-derived membranes and endocytic vesicles (5, 24, 25). The enrichment of endocytic vesicles is, however, 2- to 3-fold higher in LDMF compared to the IMF (24), whereas an opposite distribution was found for the acidification activity (Fig. 2). The pattern of distribution of acidification activity in the microsomal subfractions is indeed very similar to that of adenylate cyclase, a marker enzyme for blood-sinusoidal hepatocyte plasma membranes. When highly purified plasma membrane fractions were analyzed for ATP-dependent acidification activity, very low activities were, however, detected. Thus, the acidification activity detected in the microsomal subfractions may either originate from vesicles of the exocytic pathway, containing plasma membrane proteins destined for insertion into the cell surface membrane, or alternatively originate from endocytic vesicles containing internalized or recycling plasma membrane proteins.

In the nodular IMF and LDMF fractions very low acidification activities could be detected, supporting the idea that the acidic vacuolar compartments in nodular cells are severely deficient in capacity for acidification.

It is tentative to suggest that the reduced vacuolar acidification in nodules is responsible for the altered regulation of transferrin and asialoglycoprotein receptor-mediated functions that we and others have previously observed (6–8, 41). The evidence of a defective vacuolar acidification mechanism in nodules may also explain the hydrolase deficiency of nodular lysosomes with the concomitant reduction in intracellular proteolysis that has recently been described (42), in analogy with the situation when cells are exposed to weak bases resulting in increased secretion of lysosomal hydrolases (43, 44).

Like the mutant cells incapable of vacuolar acidification, the drug-resistant nodular cells exhibit a low uptake of iron, are iron deficient, and express low amounts of iron-containing enzymes such as cytochrome P-450, cytochrome b₅, catalase, and ribonucleotide reductase (45–47). It is reasonable to assume that the effects of iron deficiency in these cells are important for their biological behavior and probably for their resistance to cytotoxic influences. It has furthermore been reported that nodular cells overexpress multidrug resistance (mdr or P-glycoprotein) gene products (48, 49). The P-glycoprotein appears to function as a drug-efflux pump and has recently been shown to possess ATPase activity (50).

In recent studies, the acidic pH environment of intracellular endocytic compartments has in fact been implicated in the stimulation of cell proliferation by growth factors and serum (51, 52). The expression of transferrin receptors and the receptor interaction with differric transferrin for iron uptake and transplasma oxidation reduction functions are also intimately related to cell proliferation (53). Alterations in transferrin receptor levels, secondary to reduced iron uptake caused by inability to acidify vacuolar compartments in the cell, may therefore affect the growth properties and growth regulation of nodular cells and thereby influence the carcinogenic process. The biological significance of the deficiency in vacuolar acidification of nodular cells as reported in the present study and the possible relation of this defect to the drug-resistant phenotype of these cells and/or their altered mechanisms of growth are intriguing issues for further studies.
REFERENCES


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